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The Wilms' tumor gene *Wt1* is required for normal development of the retina

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The Wilms' tumor gene *Wt1* is known for its important functions during genitourinary and mesothelial formation. Here we show that *Wt1* is necessary for neuronal development in the vertebrate retina. Mouse embryos with targeted disruption of *Wt1* exhibit remarkably thinner retinas than age-matched wild-type animals. A large fraction of retinal ganglion cells is lost by apoptosis, and the growth of optic nerve fibers is severely disturbed. Strikingly, expression of the class IV POU-domain transcription factor Pou4f2 (formerly Brn-3b), which is critical for the survival of most retinal ganglion cells, is lost in *Wt1*$^{-/-}$ retinas. Forced expression of *Wt1* in cultured cells causes an up-regulation of Pou4f2 mRNA. Moreover, the Wt1(–KTS) splice variant can activate a reporter construct carrying 5′-regulatory sequences of the human *POU4F2*. The lack of Pou4f2 and the ocular defects in *Wt1*$^{-/-}$ embryos are rescued by transgenic expression of a 280 kb yeast artificial chromosome carrying the human *WT1* gene. Taken together, our findings demonstrate a continuous requirement for *Wt1* in normal retina formation with a critical role in Pou4f2-dependent ganglion cell differentiation.

Keywords: apoptosis/POU-domain factors/retina development/retinal ganglion cells/Wilms' tumor gene

Introduction

The Wilms' tumor gene *WT1* (*Wt1* in mice) was originally identified on the basis of its mutational inactivation in 10–15% of all Wilms' tumors (Hastie, 1994 and references therein). Wilms' tumor of the kidney (nephroblastoma) is the most common solid childhood malignancy, affecting around 1 in 10 000 children (Matsunaga, 1981). Nephroblastoma is a good example of malignant cell growth caused by a failure of the embryonic renal precursor, the metanephric blastema, to differentiate normally (Bennington and Beckwith, 1975). *WT1* encodes a zinc finger protein of the C_2H_2 type, which shares a high degree of structural homology with the early growth response (egr) family of transcription factors (reviewed in Rauscher, 1993). At least 24 different isoforms of WT1 protein exist, which are generated by a combination of alternative splicing, RNA editing and the use of alternative translation start sites (for a recent review see Lee and Haber, 2001). Experimental evidence suggests that the *WT1* splice variant which has three amino acids, lysine–threonine–serine (commonly referred as KTS), inserted between zinc fingers three and four of the molecule, plays a role in RNA processing (Englert *et al*., 1995b; Larsson *et al*., 1995; Davies *et al*., 1998; Landomery *et al*., 1999). The WT1(–KTS) isoform binds with high affinity to GC- and TC-rich DNA consensus sequences and functions as a transcriptional regulator (reviewed in Lee and Haber, 2001). Candidate target genes that are activated by WT1(–KTS) include the cyclin-dependent kinase inhibitor $p21^{Cip1}$ (Englert *et al*., 1997), *Amphiregulin* (Lee *et al*., 1999), *Vitamin D receptor* (Maurer *et al*., 2001; Wagner *et al*., 2001) and *Podocalyxin* (Palmer *et al*., 2001), amongst others. By generating mouse strains in which the Wt1(–KTS) and (+KTS) variants have been specifically removed, it was demonstrated recently that these two isoforms exert distinct functions during sex determination and nephron formation (Hammes *et al*., 2001).

Besides its tumor suppressor function, Wt1 also exerts important roles in embryonic development. Thus, targeted inactivation of *Wt1* in mice caused a lack of formation of the kidneys, gonads, spleen and adrenal glands, in addition to mesothelial defects (Kreidberg *et al*., 1993; Herzer *et al*., 1999; Moore *et al*., 1999). *Wt1*$^{-/-}$ embryos die *in utero*, presumably from heart failure due to disturbed cardiac growth (Kreidberg *et al*., 1993; Moore *et al*., 1999). Based on its spatio-temporal expression pattern (Pritchard-Jones *et al*., 1990; Pelletier *et al*., 1991) and the characteristic phenotype of the *Wt1*$^{-/-}$ embryos (Kreidberg *et al*., 1993), Wt1 was proposed to exert its unique role in development by allowing cells to flip back and forward between a mesenchymal and epithelial state (Moore *et al*., 1999).

It is noteworthy that Wt1 was also detected in populations of neurons in the brainstem and spinal cord (Armstrong *et al*., 1993; Rackley *et al*., 1993). These latter observations raise the interesting possibility that Wt1 is involved in the differentiation of ectodermally derived neuronal tissues. However, experimental evidence to support a role for *Wt1* in neuron development has not yet been provided. Notably, patients with Wilms' tumor are at an increased risk for developing ocular disorders including aniridia and, less frequently, optic nerve hypoplasia (Nelson *et al*., 1984; Bickmore and Hastie, 1989). These eye abnormalities in Wilms' tumor patients

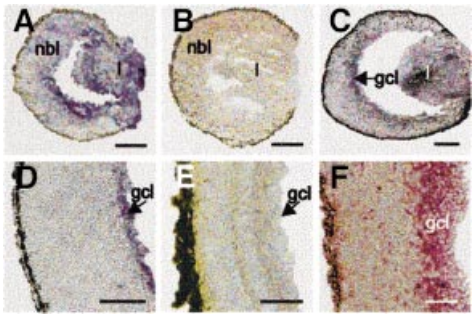

Fig. 1. Representative micrographs of tissue sections of wild-type ($Wt1^{+/+}$) eyes at different stages of development. *Wt1* transcripts are detected by mRNA *in situ* hybridization in the lens vesicle and retinal neuroblasts of E12 mice (**A**), as well as in the developing ganglion cell layer of E15 (**C**) and P1 eyes (**D**). No *Wt1* mRNA is visible in the retinas of adult mice (**E**) and in retinal tissue of E12 embryos hybridized with digoxigenin-labeled *Wt1* sense RNA strand (**B**). Note, that WT1 protein is detected by immunohistochemistry in the inner portion of the retina obtained at autopsy of a 19-week-gestation human embryo (**F**). l, lens; nbl, neuroblast layer; gcl, ganglion cell layer. Scale bars, 100 μm.

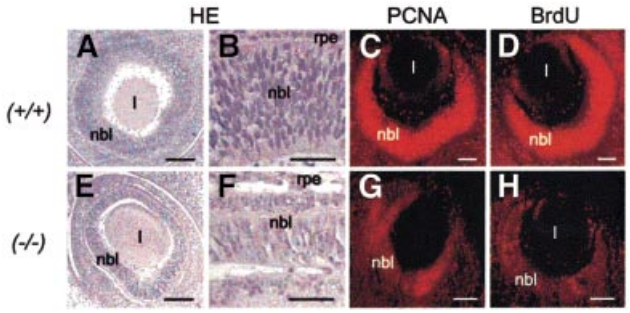

Fig. 2. Representative (immuno)histology of wild-type (+/+) and *Wt1* null mutant (–/–) eyes at E12. HE staining reveals notably thinner retinas in $Wt1^{-/-}$ (**E** and **F**) than in wild-type embryos (**A** and **B**). Immunofluorescent labeling of PCNA is clearly reduced in the mutant (**G**) versus $Wt1^{+/+}$ (**C**) retina. Incorporation of BrdU as an estimate of DNA synthesis is also decreased in $Wt1^{-/-}$ (**H**) compared with wild-type (**D**) eyes. l, lens; nbl, neuroblast layer; rpe, retinal pigment epithelium. Scale bars, 100 μm (A, C–E, G and H) and 50 μm (B and F), respectively.

are thought to result from concomitant disruption of the aniridia gene *PAX6*, which lies telomeric of *WT1* on human chromosome 11p13 (Ton *et al*., 1991). A preliminary report on the expression of *Wt1* in the developing eye is remarkable as it points towards a possible role for *Wt1* in ocular development (Armstrong *et al*., 1993).

In this study, we addressed the question of whether the Wilms' tumor gene *Wt1* is required for the differentiation of neurons in the vertebrate retina. Indeed, our findings demonstrate that *Wt1* is a critical gene for normal retina formation with impact on the proliferation of retinal progenitors and ganglion cell development.

Results

Wt1 mRNA and protein is expressed in the developing retina

We examined a total of >80 tissue sections, which were obtained from four different mouse retinas, each at the indicated developmental stages. Representative examples of *Wt1* expression in the developing retina identified by *in situ* mRNA hybridization and immunohistochemistry are shown in Figure 1. *Wt1* transcripts were initially detected throughout the neural retina and in the developing lens vesicle of 12-day-old (E12) C57BL/6 (B6) mouse embryos (Figure 1). Between E15 and the day after birth (P1), *Wt1* expression became restricted to the presumptive retinal ganglion cell layer and was absent from the retinas of adult mice (Figure 1). *Wt1* expression in the developing retinas of mice was confirmed by the detection of WT1 immunoreactivity in the future retinal ganglion cell layer of an autopsied 19-week-gestation human embryo (Figure 1).

Proliferation of the progenitor cells is impaired in $Wt1^{-/-}$ retinas

To examine whether *Wt1* is important for normal retinal development, we compared the morphology of wild-type and $Wt1^{-/-}$ eyes at different embryonic stages. Forty retinal sections that were obtained from four different normal and $Wt1^{-/-}$ mutant E12 embryos each [C57BL/6 (B6) mouse strain] were analyzed. Representative tissue sections are shown in Figure 2. $Wt1^{-/-}$ mice at E12 had markedly thinner neuroretinas, which contained significantly fewer cells than those of age-matched $Wt1^{+/+}$ embryos (Figure 2). Compared with the wild-type animals at E12, immunostaining of proliferating cell nuclear antigen (PCNA) was weaker, and incorporation of 5-bromo-2′-deoxyuridine (BrdU) was decreased in the $Wt1^{-/-}$ retinas (Figure 2). Counting of three retinal sections from BrdU-injected mouse embryos at stage E12 ($n = 3$ each) revealed that ~10 times fewer BrdU-positive cells were contained in $Wt1^{-/-}$ than in wild-type retina. Reduced cell numbers were also observed in the E12 retinas of $Wt1^{-/-}$ embryos in the MF1×B6 mouse background (see below). However, the phenotypic changes of the mutant retinas were less severe in MF1×B6 than in C57BL/6 (B6) embryos at stage E12 (data not shown). The retinal morphology of heterozygous ($Wt1^{+/-}$) embryos at E12 was normal and could not be distinguished from that of wild-type animals (data not shown).

Lack of Wt1 causes defects of retinal ganglion cells and optic nerves

Disruption of the *Wt1* gene in mice with a C57BL/6 (B6) genetic background caused embryonic lethality around mid-gestation (Kreidberg *et al*., 1993). To analyze the role of *Wt1* in retina formation at later developmental stages, we made use of the MF1×B6 mouse strain, in which a certain percentage of $Wt1^{-/-}$ embryos survive until birth (Herzer *et al*., 1999). In comparison with wild-type MF1×B6 mice, the eyes of the $Wt1^{-/-}$ mutants were notably reduced in size at E18 (1403 ± 30 μm maximum diameter in $Wt1^{-/-}$ versus 1735 ± 29 μm in $Wt1^{+/+}$ embryos, $n = 5$ each, $P < 0.0001$, Wilcoxon test). The eyes of heterozygotes were phenotypically normal and indistinguishable from those of wild-type embryos (data not shown).

Ganglion cells are among the first to appear in the embryonic mouse retina, most of them between E13 and E16 (Sidman, 1961; Cepko *et al*., 1996). To explore whether *Wt1* is critical for normal ganglion cell differentiation, we performed serial sections through the eyes of

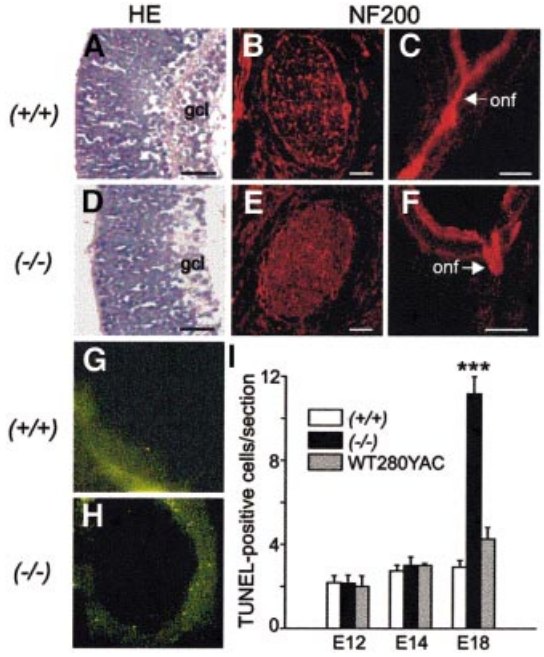

Fig. 3. Morphology of wild-type (**A**–**C**) and *Wt1*$^{-/-}$ mutant (**D**–**F**) retinas at E18. A significant (~40%) reduction in the number of ganglion cells is detected by HE staining in the E18 retinas of *Wt1*$^{-/-}$ (D) compared with wild-type (A) embryos. Immunofluorescent labeling of NF200 reveals blind-ending optic nerve fibers exiting from the *Wt1*$^{-/-}$ eyes (F). NF200 immunoreactivity is absent from cross-sections of optic nerves made at a distance of ~400 μm beyond the optic disc level (E). For comparison, NF200-positive axon bundles are clearly visible in the optic nerves of wild-type retina (B). Shown are representative results from five different embryos in both groups. gcl, ganglion cell layer; onf, optic nerve fibers. Scale bars, 50 μm (A and D), 100 μm (B and E) and 200 μm (C and F). Representative examples of apoptotic cells identified by TUNEL assay in the E18 retinas of wild-type (**G**) and *Wt1*$^{-/-}$ (**H**) mice. The majority (~60%) of the TUNEL-positive cells are located in the developing ganglion cell layer of the mutant retina (H). (**I**) The number of apoptotic cells identified by TUNEL assay in the neural retinas of wild-type (*Wt1*$^{+/+}$), null mutant (*Wt1*$^{-/-}$) and *WT280*-YAC transgenic embryos at different stages of development. An ~4-fold increase in apoptotic cells is counted in mutant versus *Wt1*$^{+/+}$ retinas at E18. Note that the average number of apoptotic cells in the developing retina is not significantly different between wild-type and *WT280*–YAC embryos. Five animals were studied in each group at the indicated time points. Five 10 μm retinal sections were made from each embryo close to the optic disc level. Values shown are means ± SEM. Asterisks indicate statistical significance (P <0.001, ANOVA with Bonferroni as post-hoc test).

wild-type and *Wt1*$^{-/-}$ E18 embryos (n = 5 each). Histological examination of hematoxylin–eosin (HE)-stained tissue sections revealed that ~40% fewer cells were contained in the future ganglion cell layer of the mutant retinas compared with that of *Wt1*$^{+/+}$ mice (Figure 3A and D). TUNEL labeling was performed to explore whether the diminished number of ganglion cells was due to premature death in the *Wt1*$^{-/-}$ retinas. Indeed, the counts of TUNEL-positive cells in the E18 retinas of *Wt1*$^{-/-}$ embryos were increased ~4-fold compared with age-matched *Wt1*$^{+/+}$ mice (n = 5 each) (Figure 3I). TUNEL labeling of tissue sections indicated that ~60% of the apoptotic cells were located in the developing ganglion cell layer of the *Wt1*$^{-/-}$ retinas (Figure 3H). Apoptosis was not enhanced in the *Wt1*$^{-/-}$ retinas prior to E18 (Figure 3I).

Each ganglion cell in the retina forms a single axon, which exits the eye as part of the optic nerve. To establish a second independent criterion of retinal ganglion cell defects, we compared the optic nerves of wild-type and *Wt1* null mutant embryos at E18. A polyclonal antibody against neurofilament (NF) 200 was used to mark the retinal ganglion cell axons and optic nerve fibers. Staining of NF200 revealed that outgrowth of axon processes from the retinal ganglion cells was severely disturbed in the *Wt1*$^{-/-}$ embryos. Indeed, the *Wt1* null mutants had abruptly terminating optic nerve fibers that extended no further than some 100 μm beyond the optic disc level (Figure 3F). Disrupted axon growth in the *Wt1*$^{-/-}$ embryos is also demonstrated by the lack of specific NF200 immunofluorescence on cross-sections made through the optic nerves at a distance of ~400 μm beyond their exit from the eyes. Normal NF200 staining of the optic nerve fibers was seen in heterozygous embryos at E18 (data not shown). Since *Wt1*$^{-/-}$ mutants normally die before end-gestation (Kreidberg *et al*., 1993; Herzer *et al*., 1999), their retinal morphology could not be analyzed postnatally.

The class IV POU-domain factor Pou4f2 is absent from the Wt1$^{-/-}$ retinas

The three closely related class IV POU-domain genes *Pou4f1*, *Pou4f2* and *Pou4f3* (formerly *Brn-3a*, *Brn-3b* and *Brn-3c*) are thought to exert distinct functions in the cell-fate specification of sensory neurons (Xiang *et al*., 1998). Selective loss of ~70% of retinal ganglion cells and reduced optic nerve diameters were reported in mice that lack the transcription factor Pou4f2 (Erkman *et al*., 1996; Gan *et al*., 1996). To explore whether *Wt1* is necessary for *Pou4f2* expression in the developing retina, we performed immunohistochemistry on tissue sections from wild-type and *Wt1*$^{-/-}$ retinas at E18 (n = 5 each). A representative immunostaining of Pou4f2 is shown in Figure 4. In agreement with previous studies (Xiang *et al*., 1993; Gan *et al*., 1996; Xiang, 1998), Pou4f2 immunoreactivity was detected in the developing ganglion cell layer of normal E18 mice (Figure 4B). However, Pou4f2 was clearly absent from the *Wt1*$^{-/-}$ retinas at E18 (Figure 4E), and also could not be detected at earlier embryonic stages in the *Wt1* mutant retinas (our unpublished observation). *In situ* labeling of Pou4f2 protein in the developing retinal ganglion cell layer was confirmed at the mRNA level. Thus, *Pou4f2* transcripts were identified by RT–PCR in the E18 retinas of normal mice, but could not be amplified in retinal tissue obtained from *Wt1*$^{-/-}$ embryos at E18 (n = 5 each) (Figure 4H). For comparison, expression of two other POU-domain family members, *Pou4f1* and *Pou4f3*, seemed normal in the *Wt1* null mutant E18 retinas (Figure 4D and F). These observations suggest that *Wt1* is required for the expression of *Pou4f2* in the embryonic retina.

WT1 activates the POU4F2 gene

Next we investigated whether Wt1 can directly activate the *POU4F2* gene. For this purpose, we examined whether forced expression of *Wt1* stimulates expression of the endogenous *POU4F2* gene in cultured cells. Furthermore, it was tested whether the activity of the *POU4F2* promoter is enhanced by co-expression of *Wt1*. A native *Wt1*-expressing cell line, which is derived from the neural

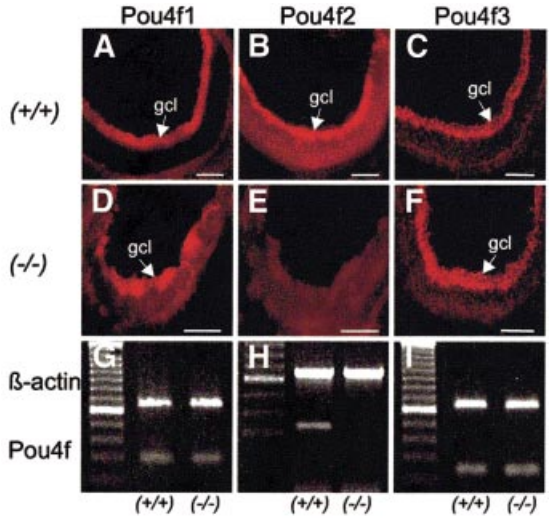

Fig. 4. Expression of *Pou4f* genes in retinal ganglion cells of wild-type (+/+) and *Wt1* null mutant (–/–) retinas at E18. Negative immunofluorescent staining of the $Wt1^{-/-}$ retina (**E**) indicates lack of expression of the class IV POU-domain transcription factor Pou4f2. RT–PCR demonstrates absence of *Pou4f2* mRNA from the E18 retinas of $Wt1^{-/-}$ embryos (**H**). No differences are seen in *Pou4f1* and *Pou4f3* expression (mRNA and protein) between wild-type and $Wt1^{-/-}$ retinas. Shown are representative data obtained from five different embryos each. gcl, ganglion cell layer. Scale bars, 100 µm. A 100 bp DNA ladder was used to estimate the sizes of the expected PCR products. The predicted lengths of the amplified targets are 274 bp (*Pou4f1*), 300 bp (*Pou4f2*), 229 bp (*Pou4f3*) and 642 bp (*β-actin*), respectively.

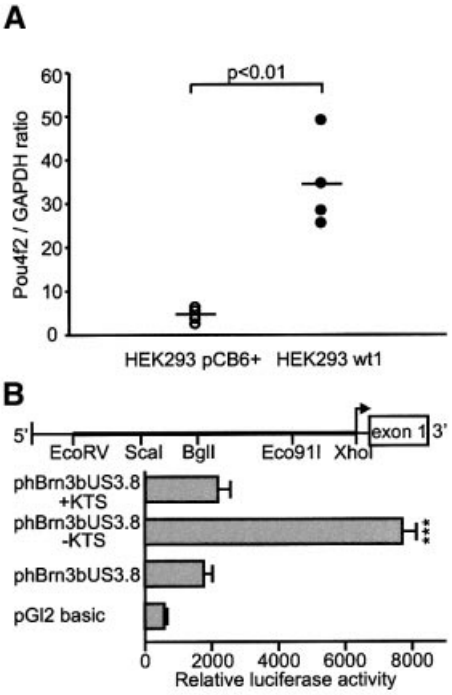

Fig. 5. (**A**) Expression of *Pou4f2* in HEK293 cells stably transfected either with a Wt1(–KTS) expression construct or with the empty pCB6+ vector. *Pou4f2* mRNA levels were quantified by real-time RT–PCR using the light cycler system (Roche Molecular Biochemicals) and normalized for *GAPDH* transcripts. Shown are the data obtained from four independent clones each of pCB6+ and Wt1(–KTS) transfected cells. Note that stable expression of Wt1(–KTS) increased *Pou4f2* mRNA levels in HEK293 cells ~8-fold. The horizontal bars indicate the mean values in each group. (**B**) Relative luciferase activities measured in the lysates of U2OS human osteosarcoma cells. U2OS cells were transiently co-transfected with phBrn3bUS3.8, *Wt1* expression constructs encoding two different splice variants (+KTS/–KTS isoforms), and a cytomegalovirus-promoter driven β-galactosidase expression vector that was used for normalization of transfection efficiencies. Plasmid phBrn3bUS3.8 contained an ~3.8 kb *Eco*RV–*Xho*I genomic sequence from the 5′-regulatory region of the human *Pou4f2* gene (schematic drawing) in the pGL2 basic reporter vector. Values shown are means ± SEM of $n = 7$ experiments each performed in duplicate. $P < 0.05$ was considered statistically significant (ANOVA).

retina, does not exist to our knowledge. We therefore made use of the previously established human embryonic kidney (HEK) 293 cell line to compare *POU4F2* mRNA levels in cells that were stably transfected either with a Wt1(–KTS) expression construct or with an empty pCB6+ vector (Wagner *et al*., 2001). Real-time RT–PCR (Roche Molecular Biochemicals) was performed with total RNA isolated from four independent clones each of *Wt1*-expressing and non-expressing HEK293 cells. *POU4F2* transcripts were normalized to *GAPDH* mRNA in each sample. Notably, HEK293 cells that stably expressed the Wt1(–KTS) isoform had on average 8-fold higher *POU4F2* mRNA levels than the pCB6+-transfected cells (Figure 5A). We isolated ~3.8 kb of the 5′-regulatory region of the human *POU4F2* gene from a BAC clone (RZPD clone ID RPCIB753D12667Q2) and ligated it into the pGL2basic reporter plasmid (Figure 5B). Sequence analysis of this construct, which was designated phBrn3bUS3.8, revealed a high GC content and several predicted WT1 consensus binding sites in the 5′-upstream region of the human *POU4F2* gene. Plasmid phBrn3bUS3.8 was transiently introduced into U2OS human osteosarcoma cells along with expression constructs encoding either of the Wt1(+KTS) and Wt1(–KTS) isoforms (Haber *et al*., 1991). The U2OS cell line has previously been used as a tool for identifying other candidate target genes of Wt1 (Lee *et al*., 1999). Basal activity of phBrn3bUS3.8 was enhanced >4-fold by the Wt1(–KTS) variant (Figure 5B). However, the Wt1(+KTS) isoform, which is thought to act at the post-transcriptional level (Englert *et al*., 1995; Larsson *et al*., 1995; Davies *et al*., 1998; Landomery *et al*., 1999), had no significant effect on reporter gene activity (Figure 5B). These findings show that the Wt1(–KTS) isoform can stimulate gene transcription from the putative *POU4F2* promoter, suggesting that *POU4F2* is a direct transcriptional downstream target gene of Wt1.

Retinal defects of the $Wt1^{-/-}$ embryos are rescued by transgenic YAC complementation

Finally, we investigated whether transgenic delivery of a yeast artificial chromosome (YAC) carrying the human *WT1* gene was able to rescue the ocular phenotype of the *Wt1* null embryos. We have recently established YAC transgenic mouse lines expressing a *LacZ* reporter gene under the control of the human *WT1* locus (Moore *et al*., 1998). *LacZ* expression in these lines recapitulated the endogenous pattern of *Wt1* (Moore *et al*., 1998), including expression in the embryonic retina (A.W.Moore, personal communication). Transgenic lines were generated by pronuclear injection of amplified YAC DNA spanning 280 kb of the human *WT1* locus (Moore *et al*., 1998). Notably, no retinal defects were visible in the *WT280*

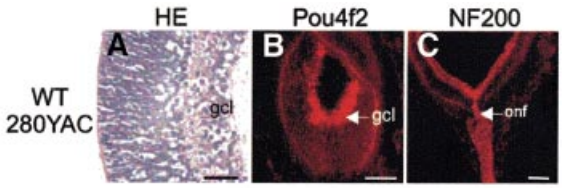

Fig. 6. Rescue of retinal phenotype by YAC complementation of the human *WT1* gene in *Wt1*$^{-/-}$ E18 embryos. Note that normal histology of the developing retina (**A**), as well as Pou4f2 immunoreactivity in the ganglion cell layer (**B**) and optic nerve fiber growth (**C**), are re-established by transgenic expression of a 280 kb YAC construct carrying the human *WT1* gene in *Wt1*$^{-/-}$ embryos. The eyes of *WT280*–YAC transgenic embryos are indistinguishable by morphological means from their wild-type counterparts at stage E18. gcl, ganglion cell layer; onf, optic nerve fibers. Scale bars, 50 μm (A), 100 μm (B) and 200 μm (C), respectively.

transgenic embryos at E18 (Figure 6). In particular, *Pou4f2* expression and optic nerve fiber growth were restored in the eyes of *WT280*–YAC transgenic embryos (Figure 6). Normal morphology of the *WT280*–YAC retinas is also reflected in a low number of apoptotic cells, which was not significantly different from that of *Wt1*$^{+/+}$ retinas at stage E18 (Figure 3I).

Discussion

In this study, we demonstrate for the first time that the Wilms' tumor gene *Wt1* encodes a regulator of neuron development. The abnormalities in the *Wt1*$^{-/-}$ mutant retinas indicate that *Wt1* is required for at least two critical functions during retinogensis: (i) proliferation of the progenitor cells; and (ii) development of the retinal ganglion cells.

Normal cell proliferation is one major determinant of retinal development. The reduced cell numbers and the weaker PCNA and BrdU stainings of the mutant retinas suggest that *Wt1* is important for normal neuroblast division during early retinogenesis. Stimulation of cell proliferation by WT1 was demonstrated previously in hematopoietic cells (Yamagami *et al*., 1996). Thus, inhibition of endogenous WT1 by treatment with antisense oligonucleotides arrested K562 leukemia cells in the G_2/M phase, suggesting that WT1 is critical for normal progression through the cell cycle (Yamagami *et al*., 1998). On the other hand, inducible expression of *Wt1* in osteosarcoma cell lines triggered an initial G_1 phase arrest that was followed by apoptosis (Englert *et al*., 1995a). These findings suggest that the effect of WT1 on cell proliferation and growth may be cell type specific and depend on additional co-factors that are differentially expressed between tissues. It is interesting to note that hypocellularity of the *Wt1*$^{-/-}$ retinas at E12 was less severe in embryos on a mixed MF1×B6 genetic background than in C57BL/6 (B6) mice (our unpublished observation). This observation is in agreement with our previous study on the role of *Wt1* in spleen development, indicating that the penetrance of the *Wt1*$^{-/-}$ phenotype depends on the existence of one or more modifier gene(s) (Herzer *et al*., 1999).

Severe abnormalities were also found in the *Wt1*$^{-/-}$ retinas at later stages of development. Consistent with the expression of *Wt1* in the inner portion of the *Wt1*$^{+/+}$ retina, a significant fraction of cells were lost by apoptosis in the developing retinal ganglion cell layer of mutant E18 embryos. Defects in the remaining ganglion cells in the *Wt1*$^{-/-}$ retinas are indicated by the failure of these cells to give rise to normally growing optic nerve fiber bundles. This phenotype of the *Wt1*$^{-/-}$ retinas is reminiscent of the abnormalities seen in mice, which lack the POU-domain transcription factor *Pou4f2* (also known as *Brn-3b*) (Erkman *et al*., 1996; Gan *et al*., 1996; Wang *et al*., 2000). In fact, several important findings of this study suggest that *Pou4f2* is among the candidate genes that mediate the effects of Wt1 in retinogenesis. First, Pou4f2 (mRNA and protein) is lost in the *Wt1*$^{-/-}$ retinas. Secondly, Pou4f2 expression and the retinal defects are rescued by transgenic delivery of the human *WT1* gene into *Wt1*$^{-/-}$ embryos. Thirdly, endogenous *Pou4f2* transcripts in human embryonic kidney cells are up-regulated by forced expression of *Wt1*. And finally, Wt1 can transactivate the promoter of the human *POU4F2* gene. Notably, the activity of the *POU4F2* promoter was stimulated only by the Wt1(–KTS) isoform, which functions as a transcriptional regulator (reviewed in Lee and Haber, 2001). In contrast, the Wt1(+KTS) isoform, which is thought to be involved in post-transcriptional mRNA processing (Englert *et al*., 1995b; Larsson *et al*., 1995; Davies *et al*., 1998; Landomery *et al*., 1999), had no significant effect. These latter results identify *POU4F2* as a novel candidate downstream target of the Wt1 transcription factor. Notably, we found that Pou4f2 and Wt1 proteins were co-expressed in the developing retina and also in glomerular podocytes of adult mouse kidney (our unpublished observation). The highly differentiated podocytes, which have been implicated in the glomerular filtration process, constitute the only cell type that expresses *Wt1* in mature kidneys (Mundlos *et al*., 1993). Given this overlapping cellular distribution of the two proteins, it will be challenging to investigate whether interaction between Wt1 and *Pou4f2* is important for the differentiation of non-neuronal tissues.

Even though the phenotype of the *Wt1*$^{-/-}$ and *Pou4f2*$^{-/-}$ retinas at stage E18 is remarkably similar, our findings raise several controversial issues that need to be addressed in more detail. For instance, the selective loss of ~70% of retinal ganglion cells in the *Pou4f2*$^{-/-}$ retina is more severe than the 40% reduction in cell numbers that we observed in the ganglion cell layer of the *Wt1*$^{-/-}$ embryos at E18. This difference appears even more dramatic if one considers that not all cells in the ganglion cell layer of the developing retina are bona fide ganglion cells (reviewed in Cepko *et al*., 1996). In view of this seeming discrepancy, it has to be recalled that apoptotic cell death in the developing *Pou4f2*$^{-/-}$ retina was found to be variable with regard to its time of onset and duration. Thus, in one study using a *lacZ* reporter gene knocked into the mouse *Pou4f2* locus to follow the fate of *Pou4f2*-mutant retinal cells, a significant increase in apoptosis was detected in mutant retinas from E15.5 to E18.5 (Gan *et al*., 1999). Postnatal changes in apoptotic cell death were not examined in this investigation. In another study, however, enhanced apoptosis in *Pou4f2*$^{-/-}$ retinas was found at E18.5, P0 and P1, indicating that premature cell death continued during the early postnatal period (Xiang, 1998). Since the *Wt1*$^{-/-}$ embryos normally die before end-gestation, their failure to express

Pou4f2 in the retina may therefore not become fully effective in terms of reduced retinal ganglion cell survival.

Another question arising from our observations relates to the expression of *Pou4f1* and *Pou4f3* (formerly *Brn-3a* and *Brn-3c*), which seemed to be normal in the $Wt1^{-/-}$ retinas. For comparison, down-regulation of *Pou4f1* and, in particular, *Pou4f3* was reported in the $Pou4f2^{-/-}$ mutant retina (Erkman *et al*., 1996; Gan *et al*., 1996; Xiang *et al*., 1998). Furthermore, *Pou4f1*- and *Pou4f2*-expressing cells were largely coincident in wild-type retina, and most of the Pou4f3-positive cells also contained Pou4f1 and Pou4f2 (Xiang *et al*., 1995). Cross-regulation between the POU-domain factors is suggested from a recent study demonstrating enhanced *Pou4f3* expression in the developing chick retina upon retroviral transfection of each of the three *Pou4f* genes (Liu *et al*., 2001). Comparing these seeming discrepancies to our results, one has to note that most, but by far not all, *Pou4f1*- and *Pou4f3*-expressing cells were absent from the $Pou4f2^{-/-}$ retinas at E18.5 (Erkman *et al*., 1996; Gan *et al*., 1996; Xiang, 1998). The latter finding suggests that a population of retinal cells may exist in which the expression of *Pou4f1* and *Pou4f3* is regulated independently of Pou4f2 (Erkman *et al*., 1996). Thus, it is conceivable that *Pou4f1* and *Pou4f3* expression was maintained in the $Wt1^{-/-}$ retinas by activation of Pou4f2-independent signaling pathways. One also has to consider that the $Wt1^{-/-}$ and $Pou4f2^{-/-}$ mutations were performed on mouse strains with different genetic backgrounds. Therefore, the variable influence of modifier gene(s) could account for the differences in *Pou4f1* and *Pou4f3* expression between the $Wt1^{-/-}$ and $Pou4f2^{-/-}$ retinas.

Our findings may also have implications for human genetic disease. The risk of Wilms' tumor patients developing ocular disorders is increased (Nelson *et al*., 1984; Bickmore and Hastie, 1989). Aniridia and, in rare cases, optic nerve hypoplasia in these patients can result from inactivation of the aniridia gene *PAX6*, which lies telomeric of *WT1* on chromosome 11p13 (Ton *et al*., 1991). As the transcriptional mechanisms underlying retinogenesis are conserved, at least in part, among species (for a review see Jean *et al*., 1998), our observations raise the possibility that some of the eye abnormalities in patients with WAGR mutations (Wilms' tumor, aniridia, genitourinary malformations, mental retardation) may arise through effects on *WT1* itself. Notably, the morphology of the retina was only disturbed in mice that had both *Wt1* alleles inactivated, but not in heterozygous animals. Moreover, the total number of *Wt1*-expressing cells in the developing retina is relatively small compared with other organs (e.g. the kidneys), and the chance of inactivating *WT1* mutations occurring is therefore relatively low.

In summary, this is the first study to demonstrate a requirement for the Wilms' tumor gene *Wt1* in neuronal development. Our findings indicate that *Wt1* has multiple functions during formation of the vertebrate retina. During early retinogenesis, *Wt1* is necessary for continuous proliferation of the progenitor cells; at the later stages, *Wt1* rescues a subset of retinal ganglion cells from apoptosis and is critical for normal growth of the optic nerve. Finally, our results suggest that Wt1 can activate transcription of the POU-domain factor, *Pou4f2*, which mediates, at least in part, the effects of Wt1 on retinal ganglion cell survival and differentiation.

Materials and methods

Animals

A heterozygous ($Wt1^{+/-}$) breeding pair [C57BL/6 (B6) strain] was obtained from The Jackson Laboratory (Bar Harbor, ME) and genotyped according to the protocol provided. The *Wt1* mutation was crossed into the MF1×B6 genetic background as described previously (Herzer *et al*., 1999).

Histology and immunohistochemistry

Staged embryos (the morning of vaginal plug was considered E0) were fixed overnight at 4°C in paraformaldehyde [3% in phosphate-buffered saline (PBS)] and either embedded in paraffin for conventional histology or snap-frozen in pre-chilled isopentane and then embedded in Tissue-Tek® optimal cutting temperature (OCT) compound (Sakura Finetek) for immunohistochemical analysis. Notably, examination of tissue from $Wt1^{-/-}$ embryos revealed no gross pathological changes, indicating that the retinal cell defects were not simply due to an overall poor health of these animals. Four-micrometer tissue sections were cut and transferred onto gelatin-coated glass slides. Tissue sections were permeabilized with 0.1% Triton X-100 in PBS and blocked by incubation for 1 h in 10% normal goat serum [in PBS, 0.1% Triton X-100, 3% bovine serum albumin (BSA)]. Following treatment (16 h, 4°C) with primary antibody and 3 × 15 min washes in PBS, the slides were incubated for 1.5 h with biotinylated secondary antibodies (1:150 dilutions in PBS, 1% BSA; Vector Laboratories, Inc.) and streptavidin–Cy3 complex (Sigma). The slides were viewed under an epifluorescence microscope (Axioplan 2; Zeiss) connected to a digital camera (Spot RT™Slider, Diagnostic Instruments) using the Metamorph V4.1.2 software (Universal Imaging Corp.). The following primary antibodies were used: WT1 polyclonal antibody diluted 1:150 (C-19; cat.# sc-192; Santa Cruz Biotechnology), Pou4f1 monoclonal antibody diluted 1:150 (14A6; cat.# sc-8429; Santa Cruz Biotechnology), Pou4f2 polyclonal antibody diluted 1:150 (C-13; cat.# sc-6026; Santa Cruz Biotechnology), Pou4f3 polyclonal antibody diluted 1:150 (BabCO, Richmond, CA), NF200 polyclonal antibody diluted 1:150 (cat.# N 4142; Sigma), PCNA monoclonal antibody diluted 1:150 (PC10; cat.# sc-56; Santa Cruz Biotechnology), and BrdU polyclonal antibody diluted 1:150 (BMC9318; Roche Molecular Biochemicals).

Retinal ganglion cell counts

Ten-micrometer serial sections were made close to the optic disk level and stained with HE to estimate the number of cells in the developing ganglion cell layer of wild-type and *Wt1* null mutant E18 retinas. Twenty retinal sections each obtained from five different $Wt1^{+/+}$, $Wt1^{-/-}$ and WT280–YAC transgenic embryos were counted under the microscope (Axiovert S100; Zeiss) at 200-fold magnification.

In situ mRNA hybridization

Non-radioactive *in situ* mRNA hybridization was carried out on 10-μm paraformaldehyde (3% in PBS) fixed cryostat sections of staged mouse eyes as described by Braissant and Wahli (1998). Briefly, digoxigenin-labeled RNA probes were synthesized *in vitro* by transcription with T3 RNA polymerase (Roche Molecular Biochemicals) from *Bam*HI-linearized plasmid pWt1-ISH. Plasmid pWt1-ISH contained the complete mouse *Wt1* cDNA coding sequence (Buckler *et al*., 1991) on a 1.5 kb *Sau*3AI piece ligated into the blunt *Xba*I site of pBluescript II KS$^+$. Sense RNA transcripts were generated using T7 RNA polymerase after linearization of pWt1-ISH with *Not*I. Hybridization was performed for 48 h at 58°C in a 50% formamide, 5× SSC solution supplemented with 60 μg/ml salmon sperm DNA (Promega). The tissue sections were washed at high stringency (1 h in 0.1× SSC at 65°C), and mRNA hybrids were detected by incubation with alkaline phosphatase-coupled anti-digoxigenin antibody and staining with NBT/BCIP (Roche Molecular Biochemicals). Stained tissue sections were viewed under an Axiovert S100 microscope (Zeiss).

Detection of apoptotic cells

Apoptotic cells were detected by TUNEL assay in the neural retinas of paraformaldehyde-fixed mouse embryos using the *In Situ* Cell Death Detection Kit (Roche Molecular Biochemicals). Five 10 μm retinal sections near the optic disk level were obtained from each animal to

determine the number of apoptotic cells in *Wt1*$^{+/+}$, *Wt1* null mutant and *WT280*–YAC transgenic embryos. Five animals were studied in each group at the indicated time points (E12, E14, E18).

BrdU labeling and analysis

Timed pregnant mice were injected intraperitoneally with a single dose of BrdU (5 mg/100 g body weight; Roche Molecular Biochemicals). The E12 embryos were removed after 3 h from ether-anesthetized animals and fixed overnight at 4°C in a 3% paraformaldehyde–PBS solution. BrdU incorporation was detected by immunofluorescent staining of cryostat sections using anti-BrdU antibody at 1:150 dilution (BMC9318; Roche Molecular Biochemicals) according to the protocol described above.

RT–PCR and real-time RT–PCR

First strand cDNA synthesis was performed with 2 µg of total RNA using oligo(dT) primers and superscript II reverse transcriptase (Life Technologies). One-tenth of the reaction product was used for PCR amplification carried out in a thermal cycler (Biometra, UNO-II, Göttingen, Germany): DNA denaturing at 94°C, primer annealing at 55°C, primer extension at 72°C. The following gene-specific oligonucleotide primers were used for PCR amplification: *Pou4f1*, 5′-CATGATCG-CGCTCAAGCCCAT-3′ (forward primer), 5′-CCGCTTCTGCTTCTG-TCTCTG-3′ (reverse primer); *Pou4f2*, 5′-GCAATATATTCGGCGGG-CTGG-3′ (forward primer), 5′-TTAGGTGCTCAAGCAGCTCGC-3′ (reverse primer); *Pou4f3*, 5′-GAAGCTTTGATGAGAGCCTGC-3′ (forward primer), 5′-GATGTGCTCAAGTAAGTCGCC-3′ (reverse primer); β-*actin*, 5′-ACCAAGGTGTGATGGTGGGAATG-3′ (forward primer), 5′-CGCTCGTTGCCAATAGTGATG-3′ (reverse primer). The expected PCR products had lengths of 274 bp (*Pou4f1*), 300 bp (*Pou4f2*), 229 bp (*Pou4f3*) and 642 bp (*β-actin*), respectively. The number of PCR cycles was adjusted to 28 to be within the exponential phase of the PCR. Real-time RT–PCR was performed with the use of the light cycler system (Roche Molecular Biochemicals) as described in detail elsewhere (Hammes *et al*., 2001). *POU4F2* mRNA obtained from the HEK293 cell line was amplified after reverse transcription using the following oligonucleotide primers: 5′-GGAGAAGAAGCGCAAGC-3′ (forward primer); 5′-TCTGGAGAGGCCAAGAGTC-3′ (reverse primer). For normalization of POU4F2 transcripts, the housekeeping gene *GAPDH* was co-amplified in each sample: 5′-AACAGCGACACCCACTCCTC-3′ (forward primer); 5′-GGAGGGGAGATTCAGTGTGGT-3′ (reverse primer).

Cloning of the 5′-regulatory region of the human POU4F2 gene

A bacterial artificial chromosome (BAC) spanning the human *POU4F2* gene (NCBI accession No. AC027193) was obtained from the German Resource Center for Genome Research (RZPD clone ID RPCIB753D12667Q2). The *Escherichia coli* DH10B host strain was grown overnight at 37°C in LB liquid medium supplemented with 20 µg/ml chloramphenicol. The BAC DNA was isolated by alkaline lysis and subsequent purification according to the manufacturer's protocol. Twenty micrograms of plasmid DNA were double digested with *Xho*I and *Eco*RV, separated on a 0.8% agarose gel, and blotted onto positively charged nylon membrane (Roche Molecular Biochemicals) with 10× SSC as a transfer buffer. Southern hybridization was performed overnight at 65°C with a ^{32}P-labeled DNA probe (PrimeIt II labeling kit; Stratagene) as described previously (Scholz *et al*., 1997). The probe consisted of a 387 bp sequence located ~2.4 kb upstream of the predicted transcription start site in the human *POU4F2* gene. The following PCR primers were used for generation of the probe: 5′-ACACAG-TGAAGCGCGGTGTCT-3′ (forward primer); 5′-GTAGCCGAAGGG-AACTCCTAG-3′ (reverse primer). The ^{32}P-labeled probe hybridized specifically with an ~3.8 kb *Eco*RV–*Xho*I genomic DNA fragment, which was identified by dideoxy sequencing of both strands, and blunt-end ligated into the *Xho*I restriction site of the pGL2basic reporter plasmid (Promega). This construct was designated phBrn3bUS3.8.

Cell transfections and reporter gene assays

Three micrograms of the phBrn3bUS3.8 reporter construct together with *Wt1* expression vectors (16 µg) and 1 µg of a cytomegalovirus-driven β-galactosidase plasmid were transiently co-transfected into U2OS osteosarcoma cells using the calcium phosphate precipitation technique (Gorman *et al*., 1982). The *Wt1* expression constructs contained cDNA encoding two alternatively spliced variants (+KTS/–KTS isoforms) in the pCB6^{+} plasmid (Haber *et al*., 1991). U2OS cells were grown to 60% confluence on 100 mm dishes in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal calf serum (Biochrom KG), 100 IU/ml penicillin (Life Technologies) and 100 µg/ml streptomycin (Life Technologies). The cells were lysed 48 h after the transfections, and luciferase reporter gene activities were measured in a luminometer (Microlite TLX1; Dynatech Laboratories Inc.) with beetle luciferin as substrate (Promega). β-galactosidase activities were determined spectrophotometrically (Beckman DU 540 spectrophotometer) using a commercial kit according to the manufacturer's instructions (Promega). Values are given as relative light units normalized to β-galactosidase activities as an internal control for transfection efficiencies. Data shown are means ± SEM of $n = 7$ experiments each performed in duplicate. $P < 0.05$ was considered statistically significant (ANOVA).

Generation and molecular characterization of YAC transgenic mice

The YAC construct *WT280*, which was used for complementation of the ocular defects in *Wt1*$^{-/-}$ mice, is described in detail elsewhere (Menke *et al*., 1998). *WT280* contained the entire human *WT1* locus (~50 kb) flanked by ~220 kb upstream and ~10 kb downstream sequence on either side. This construct was generated by truncation of a larger 470 kb clone (*WT470*) at a position ~10 kb downstream of the *WT1* termination site (Menke *et al*., 1998). Transgenic lines were obtained by injection of amplified *WT280*–YAC DNA into pronuclei of fertilized mouse oocytes as described (Schedl *et al*., 1996). Positive lines were identified by PCR and Southern blot analysis (Moore *et al*., 1999).

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